American Journal of Biology and Life Sciences
2018; 6(4): 67-74
http://www.openscienceonline.com/journal/ajbls
ISSN: 2381-3784 (Print); ISSN: 2381-3792 (Online)

Open Science

Effect of NPK Fertilizer Concentration on Growth and Lipid Accumulation of *Picochlorum* sp.

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To cite this article

Trung Vo, Quyen Nguyen, Phuc Nguyen, Dat Tran, Tran Nim, Hung Nguyen, Truc Mai. Effect of NPK Fertilizer Concentration on Growth and Lipid Accumulation of *Picochlorum* sp. *American Journal of Biology and Life Sciences.* Vol. 6, No. 4, 2018, pp. 67-74.

Received: August 28, 2018; **Accepted**: September 10, 2018; **Published**: October 19, 2018

Abstract

Microalgae are known as a major object for second generation biofuels and functional foods due to high lipid content accumulated in cells under different nutrient media. *Picochlorum* is a small, unicellular, fast growing green microalgae capable of producing high intracellular lipids. Recent studies indicated that *Picochlorum* accumulated higher lipid content with high percentage of polyunsaturated fatty acids such as arachidonic (AA), eicopentaenoic (EPA), and docosahexaenic (DHA) acid. However, nutrient sources with low cost, especially nitrogen and phosphorus were used to increase biomass and lipid content in *Picochlorum* cells to increase higher productivity in industrial culture. In this study, a low cost comercial fertilizer, NPK was used as a sourse of nitrogen and phosphorus to attain higher biomass and intracelluclar lipid content of *Picochlorum*. *Picochlorum* was cultivated in MD4 medium at different NPK fertilizer concentrations, ranging from 0.05 g/l to 1.0 g/l. The results indicated that various concentrations of NPK fertilizer has signficantly impacted on the growth, photosynthetic pigment compositions, as well as lipid content and profile of the organism. At NPK concentration of 0.1 g/l, the growth of *Picochlorum* cells was highest with specific growth rate ($\mu = 0.156$ day^{-1}) and cell density (69.5 x 106 cells/ml) after 27 days of cultivation. In addition, higher chlorophyll and carotene content were obtained in cultures grown in MD4 medium containing 0.1 – 0.15 g/L fertilizer. Lipid content per volume of *Picochlorum* increased, however lipid content per cells was the highest under NPK nutrient starvation after 12 day of cultivation (8.031 pg/cell of day 27). Growth of *Picochlorum* sp. was inhibited and significantly at fertilizer concentrations of above 0.5 g/L. Therefore, MD4 medium containing 0.1 – 0.15 g/L of NPK fertilizer concentration can be used at *Picochlorum* growth phase and the condition of NPK starvation can be used as a stress factor for lipid accumulation of *Picochlorum* cells.

Keywords

Picochlorum, NPK Fertilizer, Lipid Accumulation

1. Introduction

The genus *Picochlorum* is known as a new microalga (Trebouxiophyceae, Chlorophyta) found to have high growth rate and can thrive in adverse conditions [1], [2]. Several strains of *Picochlorum* sp. have been used as models for horizontal gene transfers and as potential sources of valuable compounds. *Picochlorum* SENEW3 (SE3) is a tiny coccoid (2–3 μm in cell diameter) green alga. The genome of *Picochlorum* SE3 contains more than 7000 genes involving in urea metabolism, acetate assimilation and fermentation, acetoin production and glucose uptake. Interestingly, there also existed horizontally transferred genes involved in stress adaption, such as osmolyte production [3], [4]. The total lipid content of *Picochlorum* sp HM1 is not particularly high, which accounts for only 23% of dry weight, but its fatty acid composition make it an ideal candidate for biodiesel production. The high content of lutein (3.5 mg g^{-1} DW) and zeaxanthin (0.4 mg g^{-1} DW) suggests that the microalga could also be a good source for natural eye vitamin supplements [1]. *Picochlorum oklahomensis* is rich in oil,

protein and polyunsaturated fatty acids, and is studied as a potential microalga strain for biofuel and bioproduct applications [5].

More investigations showed that the growth, proteins, carbohydrates and chlorophyll a and fatty acid compositions of marine microalga *Picochlorum* sp. were affected by environmental conditions. Under nutrient stresses, growth measured by cell count and biomass productivity decreased as compared by control culture after 12 days [6]. Carbohydrate and lipid contents increased in the cultures growing on medium containing only half the concentration of or complete absence of $NaNO_3$. Under phosphorus stress, the carbohydrate content increased by nearly 30.27%, 62.38%, but protein content decreased by 24.5%, 37.3% for the culture growing on medium containing only half the concentration of or complete absence of NaH_2PO_4. $2H_2O$, respectively [6]. *Picochlorum* sp. isolated from Vietnam has the best growth performance at light intensity of 50 µmol photon.m^{-2}.s^{-1} and cell density of 5x10^6 cells.ml^{-1} [7]. The highest total lipid content was found to be 48.6% per dry weight (DW), and is composed of 27.84% docosahexaenoic acid (DHA); the remaining lipids was mostly of C16 and C18 fatty acids, which is appropriate for biofuel production. In addition, the species also has a high content of essential amino acids under different culturing conditions [8]. This study aimed to investigate the growth and lipid accumulation of *Picochlorum* sp. in MD4 medium containing different NPK fertilizer concentrations as nitrogen and phosphorus supplementary source.

2. Material and Methods

2.1. Picochlorum sp. Strain and Cultural Conditions

Picochlorum sp. was obtained from the Laboratory of algal technology, International University, Viet Nam National University Ho Chi Minh City. The alga was grown in 0.5 M MD4 medium, pH 7.5 [9].

Picochlorum sp. was cultivated in 50 mL MD4 medium in 100 mL Erlenmeyer flasks containing NPK fertilizer (Dau Trau 501, product of Binh Dien - Mekong Joint Stock Company, Viet Nam) with concentrations varying from 0.05 g/L to 1.0 g/L. The control treatment does not have supplementary fertilizer. Analysis of pigments and lipid contents were carried out after 12 days of cultivation. The treatments were triplicated; cultures were grown at 25°C under continuous light intensity of 50 µmol photon.m^{-2}.s^{-1}.

2.2. Growth Analysis

100 µl of algal suspension were immobilized by Lugol solution (5% iodine and 10% potassium iodide). Cell density was determined by direct cell count every three days, using a light microscope with 0.1 mm deep counting chamber (Neubauer Haemocytometer). Cell number was determined by the following formula: Number of cells/ml = total cells counted x 10^4 x dilution factor.

2.3. Pigment Analysis

One milliliter aliquot of algal suspension was centrifuged at 10000 rpm for 5 minutes and pellets were extracted with 3ml of Ethanol:Hexane 2:1 (v/v). Two milliliters of water and 4ml hexane were added and the mixture vigorously shaken and centrifuged again at 5000 rpm for 5 minutes. The hexane layer was separated and its absorbance at 450nm, 662nm and 645nm were measured. Total carotene was calculated as A_{450} × 25.2 which is equivalent to the micrograms of carotene in sample [10], [11]. Chl *a* and Chl *b* contents were estimated according to [12]:

$$\text{Chl a (µg/ml)} = 11.75\ (A662) - 2.35\ (A645)$$

$$\text{Chl b (µg/ml)} = 18.61\ (A645) - 3.96\ (A662)$$

$$\text{Total chlorophyll} = \text{Chl } a + \text{Chl } b$$

Where: Chl *a* is chlorophyll a, Chl *b* is chlorophyll b

2.4. Lipid Determination

Sulfo-phospho-vanillin assay for lipid accumulation

Phosphovanillin reagent was prepared by initially dissolving 0.06 g vanillin in 2 ml absolute ethanol; 8 ml deionized water and stirred continuously. Subsequently 50 ml of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in the dark until use. To ensure high activity, fresh phospho-vanillin reagent was prepared shortly before every experiment run [13], [14].

For SPV reaction of the algal culture for lipid quantification, one mL of algal suspension was centrifuged at 10000 rpm for 5 minutes. Into the pellet, 2 mL of concentrated (98%) sulfuric acid was added to the sample and heated for 10 minutes at 100°C, and was cooled for 5 minutes in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added, and the sample was incubated for 15 minutes at 37°C incubator shaker at 200 rpm. Absorbance reading at 530 nm was taken in order to quantify the lipid within the sample [13], [14].

The standard curve of lipid was constructed using commercial Canola oil (Product of Tuong An Vegetable Oil Joint Stock Company; 48/5 Phan Huy Ich Street, Ward 15, Tan Binh District, Ho Chi Minh City, Vietnam). Different concentrations (10-150 µg) of standard lipid samples were prepared in clean test tubes and dissolved in chloroform (final concentration 1 mg/mL). The tubes were kept at 90°C for 10 min to evaporate the solvent and 2 mL of concentrated (98%) sulfuric acid was added to the tubes. Further sample was prepared by following SPV reaction methods.

2.5. Data Analysis

Data was processed in Excel 2013 and analyzed by one-way ANOVA using SPSS software version 20.0. All significant levels were set at $p < 0.05$.

3. Result and Discussion

3.1. The Growth of *Picochlorum* sp.

Macronutrient sources and concentrations have been tested on many microalgae such as *Chlorella* [15], *Dunaliella* [16], [17], *Haematococcus* [18], *Nanochloropsis* [15], *Scenedesmus* [19] and *Picochlorum* [6], [20] to examine growth and accumulation of organic compounds including carotenoids, lipids and proteins. Nitrogen is the most common limiting factor of growth and lipid accumulation of microalgal species [21]. In certain cases, phosphate is the limiting nutrient. The growth rate of *Haematococcus pluvialis* TMU1 increased up to 86% as well as cell density obtained the highest with the modified BBM medium containing 3-fold higher phosphate [18].

The study showed that NPK fertilizer concentrations has a significant impact on the growth of *Picochlorum* sp. ($p = 0.000$). The growth in control condition was significantly lower than in media supplemented with 0.05, 0.1, 0.15 and 0.3 g/L of fertilizer ($p = 0.000$). *Picochlorum* cells count significantly decreased after 18 days of cultivation under high fertilizer concentration (0.5 and 1.0 g/L). However, cell number was not significant different at 0.05, 0.1, 0.15 and 0.3 g/L fertilizers ($p = 0.060$). At 0.1 g/L, high cell density was reached after 9 days of cultivation, but the growth curve greatly fluctuates. Similarly, under 0.05, 0.15, 0.3 g/L NPK fertilizer concentrations, cell density obtained high and constant after 6 or 9 days of cultivation (figure 1).

Microalgal cells are frequently cultured in nitrogen rich conditions to stimulate biomass growth [22]. Previous experiment investigating the effect of different organic and inorganic N sources on the cell growth and biochemical composition of the marine microalga *Tetraselmis* sp. found that organic nitrogen sources had positive effect on growth, and inorganic ammonium, while discouraged growth, stimulated lipid production. Ammonium toxicity observed on growth was probably due to the volatilization of ammonia as pH of the media increased to 8.5-9.5 due to photosynthetic activity [21]. According to [23] the ammonium compound and nitrogen concentration affected content of different cellular constituents in *Dunaliella tertiolecta* such as protein, carbohydrate, chlorophyll a. It is thus suggested that macronutrient sources and concentration in cultural medium affected significantly on the growth and compound compositions of *Picochlorum* sp.

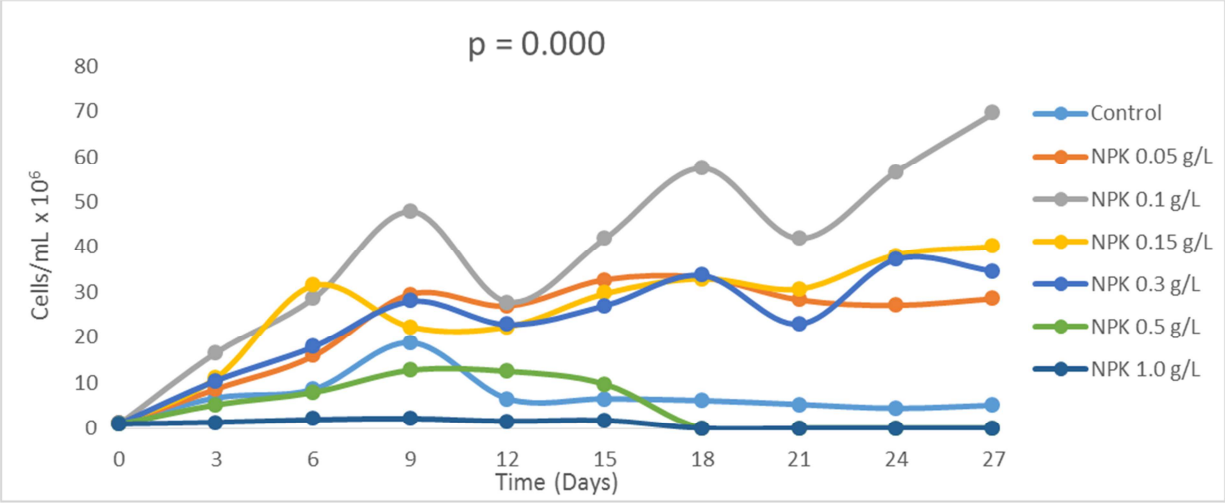


Figure 1. *The growth of Picochlorum sp. under different NPK fertilizer concentrations in MD4 medium.*

3.2. Chlorophyll Content of *Picochlorum* sp.

Macronutrients such as nitrogen and phosphorus are essential for normal growth and production of organic compounds including protein, lipid, chlorophyll in plants as well as microalgae. In microalgae, nitrogen and phosphorus limitation in cultural medium severes growth rate and induce accumulation of large number of secondary compounds such as carotenoids and lipids. Chlorophyll *a* and total chlorophyll contents of *Picochlorum* increase during cultivation. Under 0.1 g/L and 0.15 g/L NPK fertilizer concentrations, chlorophyll content (per volume and cell) increased rapidly and reached the highest level from day 21 to day 27 of the cultivation. Chlorophyll content is also significantly different among fertilizer treatments ($p = 0.000$) (figure 2, 3). Growth enhancement was in line with remarkable increase of total chlorophyll content - an essential component of photosynthesis in green algal cells [18]. Increasing demand for chlorophyll for cell growth is adequately supplied by supplemented nitrogen, as it is one of the essential factors involving in formation the porphyrin ring. Thus, MD4 medium with 0.1-0.15 g/L fertilizer supplemented successfully induced the growth of *Picochlorum* sp.

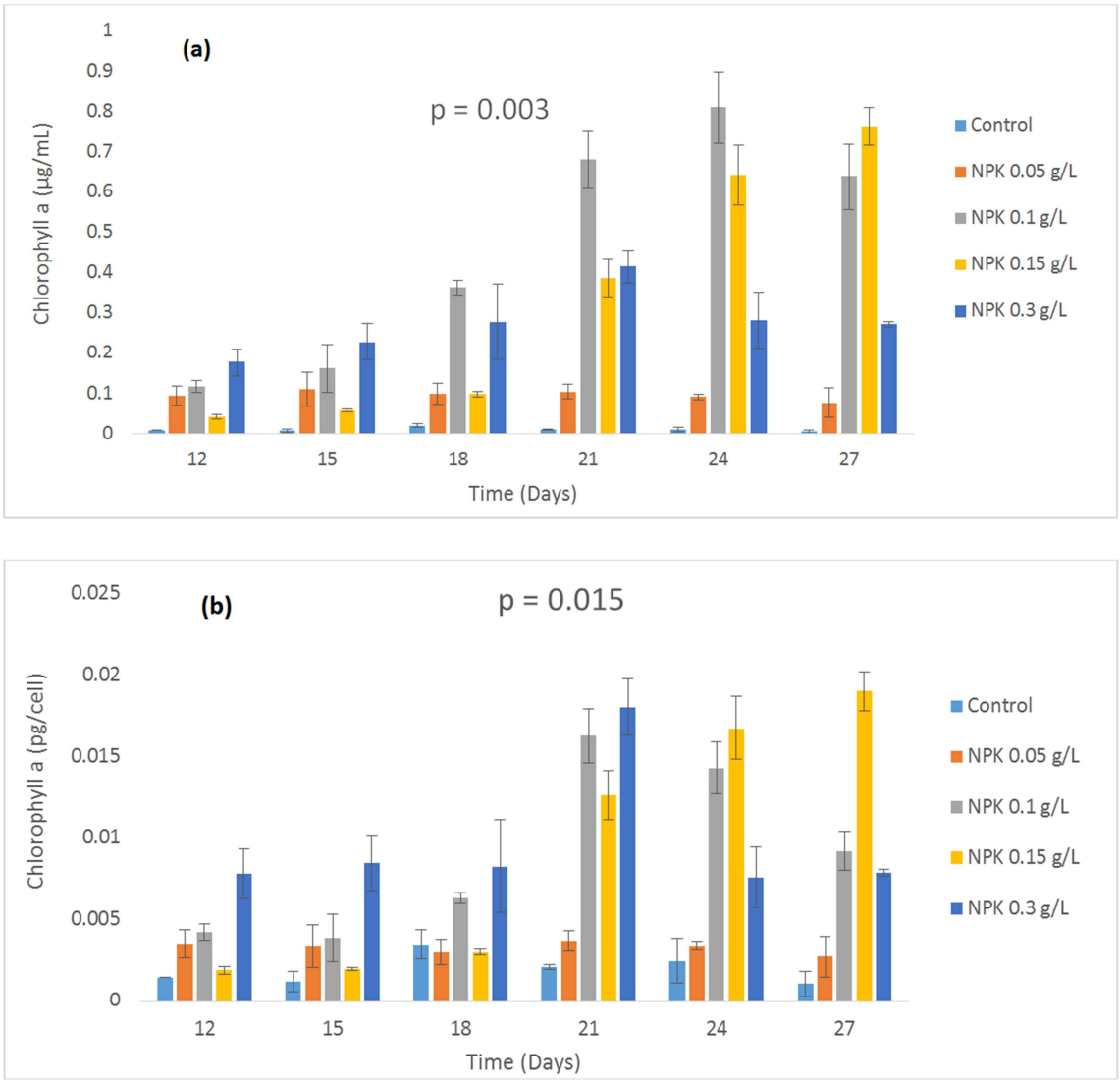
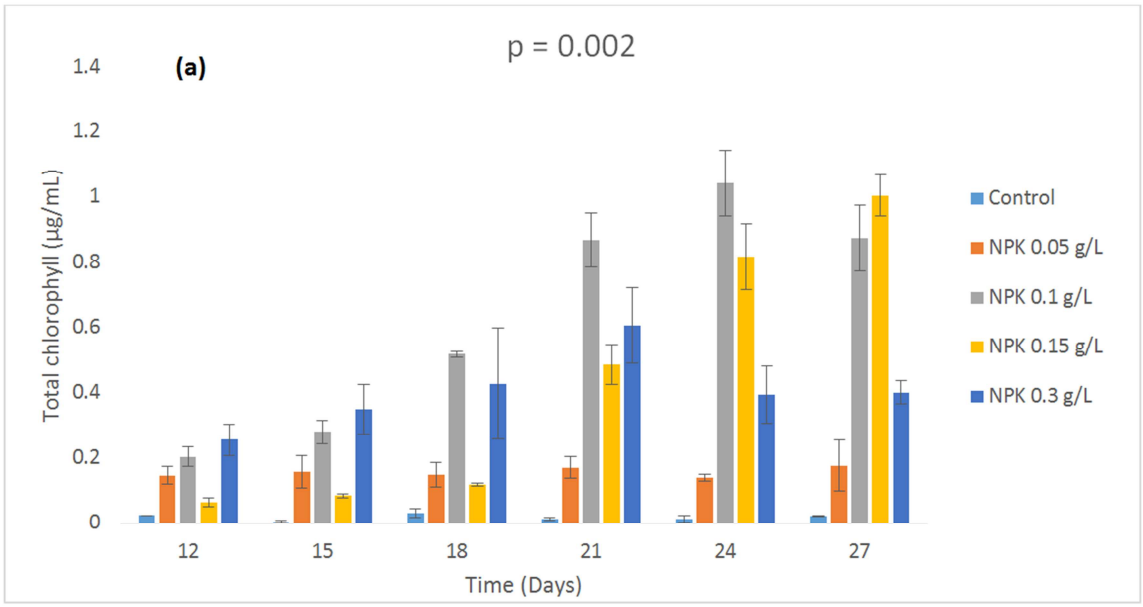


Figure 2. *Chlorophyll a content of Picochlorum sp. per mL (a) and cells (b) under different NPK fertilizer concentrations in MD4 medium.*



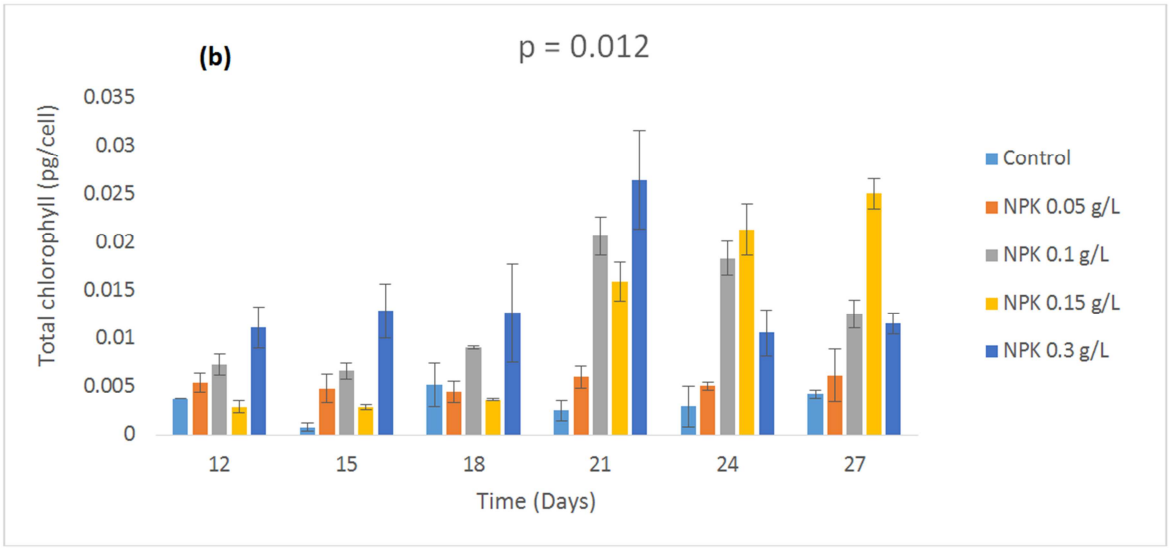
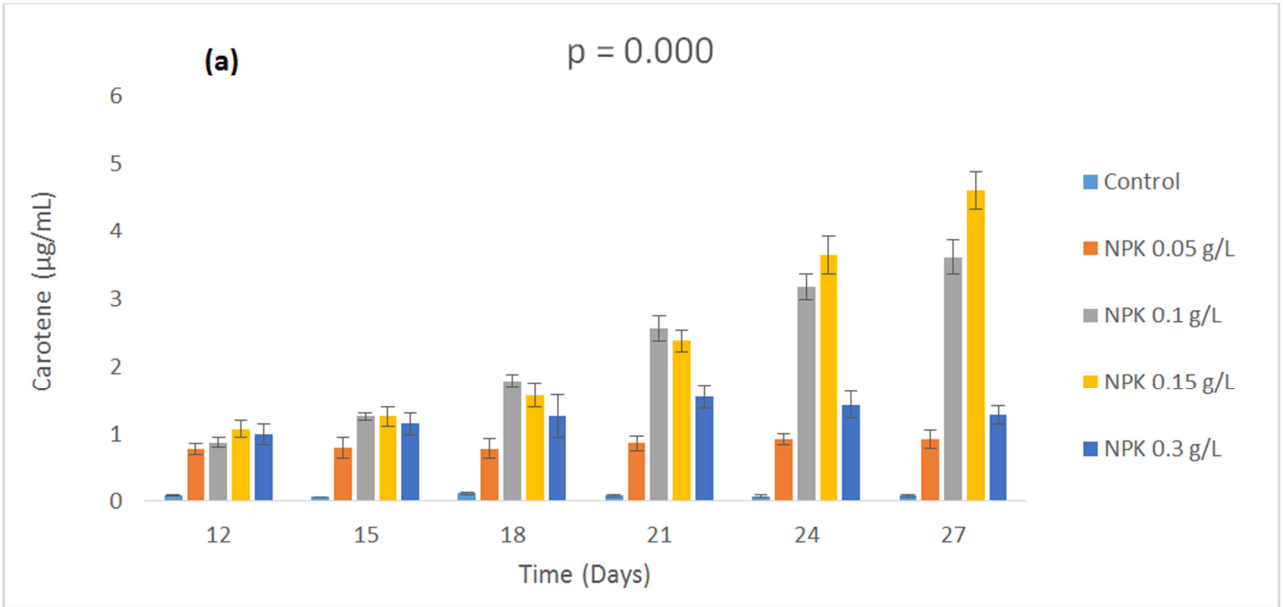


Figure 3. *Total chlorophyll content of Picochlorum sp. per mL (a) and cells (b) under different NPK fertilizer concentrations in MD4 medium.*

3.3. Carotene Content of *Picochlorum* sp.

For phototropic organisms, the synthesis of chlorophyll and carotenoids is essential for the growth and production of organic compounds. Most carotenoids are bound to membrane-bound pigment-protein complexes, such as reaction centers, light-harvesting and cytochrome b_6f complexes [24]. Figure 4 showed carotene content of *Picochlorum* (per volume and cell) during the cultivation. Carotene content of *Picochlorum* sp. increased remarkably from day 21 at 0.1 g/L and 0.15 g/L NPK fertilizer treatments and there was significant difference ($p = 0.000$). Decrease in nutrient concentration of the medium, especially nitrogen and phosphorus stimulated increase in biosynthesis of secondary carotenoid after 21 days of cultivation. Nitrogen and phosphate are two important macronutrients for growth and metabolism of algal cells. Nitrogen is a fundamental element structuring proteins and nucleic acids. Phosphate are essential macromolecules for all living cells, and are crucial for the construction of DNA and RNA backbones. Phosphorus is also a major component of phospholipids [6]. Macronutrient availability in cultural media impact growth and the ability to synthesize key compounds of microalgal cells, such as chlorophylls, carotenoids and lipids. According to [25], carotenoids production of *D. salina* cells were inhibited when grown on nitrogen supplemented media, even under exposure to unfavorable light intensity. However, under nutrient starvation, microalgal metabolic processes change and carotenoid and lipid accumulation increases rapidly.



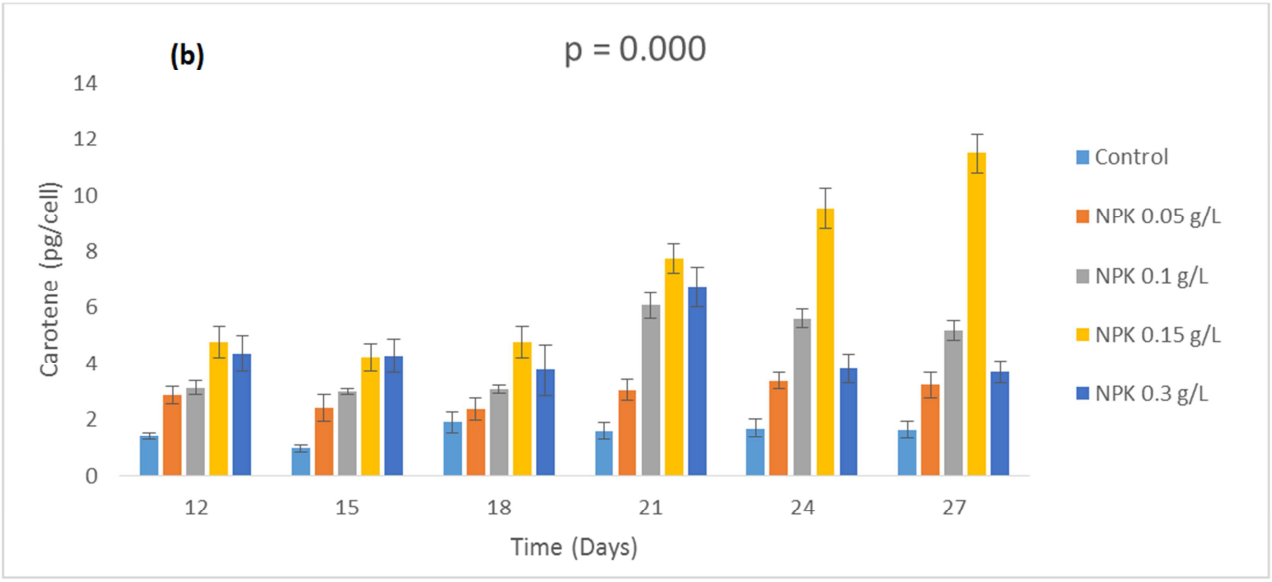
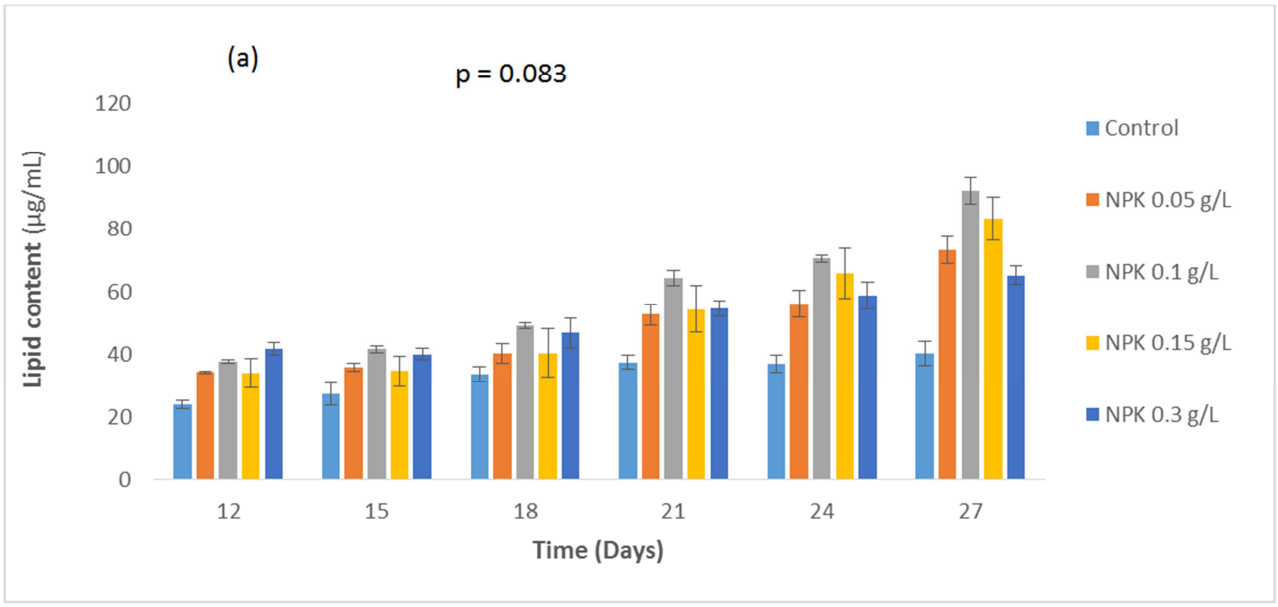


Figure 4. *Carotene content of Picochlorum sp. per mL (a) and cells (b) under different NPK fertilizer concentrations in MD4 medium.*

3.4. Lipid Accumulation of *Picochlorum* sp.

Climate changes and environmental pollution from exhaust gases, coinciding with depletion of fossil fuel sources have been occurring on a global scale. Therefore, renewable sources of energy have been rapidly exploited. Microalgae are one of the potential objects for production of biofuel, accumulate high lipid content under stressful culturing conditions. Many researches have demonstrated that *Nanochloropsis*, *Picochlorum*, *Dunaliella* and *Chlorella* were capable of lipid production with mostly C16 and C18 fatty acids desirable for biofuel production. Lipid content (per volume) of *Picochlorum* sp. increased in all NPK supplemented experiments ($p < 0.005$), while lipid content in the control experiment was not significantly different from day 12 to day 27 ($p = 0.011$) (figure 5a). However, lipid content per cells of the control experiment was higher than NPK supplemented experiments ($p = 0.000$) (figure 5b). *Picochlorum* sp. cultivated in complete nitrogen and phosphorus starvation of has decreased growth rate but increased lipid content, in particular triacylglycerol (TCG) and most saturated fatty acids. Macronutrient starvation induced biosynthesis of *de novo* triacylglycerol (TCG) as well as the conversion of present membrane lipids to TCG [6].



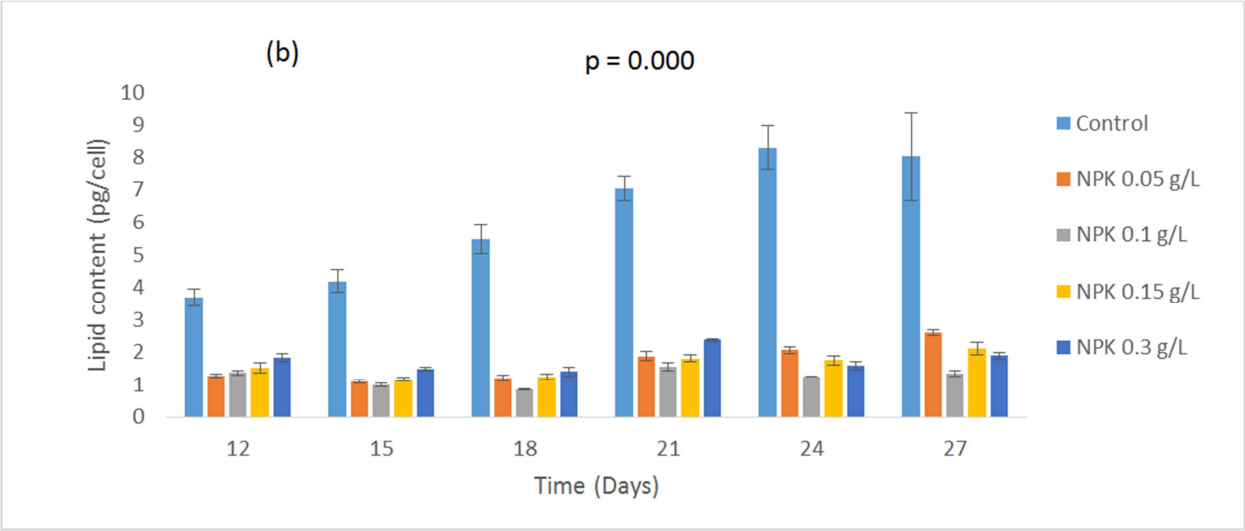


Figure 5. *Lipid content of Picochlorum sp. per mL (a) and cells (b) under different NPK fertilizer concentrations in MD4 medium.*

4. Conclusion

Picochlorum is a promising microalga for biofuel production and functional foods with rapid growth and high lipid profiles and content under different cultural conditions. NPK fertilizer as nitrogen and phosphorus sources has been commonly applied, and is cost-effective in microalgal cultivation. This study demonstrated that MD4 medium containing 0.1-0.15 g/L of fertilizer stimulated growth and synthesis of photosynthesis pigments of *Picochlorum* sp. Growth was inhibited when *Picochlorum* cells were suspended in medium containing above 0.5 g/L fertilizer. Additionally, lipid content per cells was the highest in control medium. Applying nutrient starvation after an initial growing stage supplemented with 0.1-0.15 g/L fertilizer is suggested as a strategy to increase lipid accumulation in *Picochlorum* sp.

Conflict of Interests

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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